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IN THE UNITED STATES PATENT & TRADEMARK OFFICE

IN RE APPLICATION OF :

KENJI SUGIMOTO, ET AL. : EXAMINER: J. DUNSTON

SERIAL NO: 10/718,712 :

FILED: NOVEMBER 24, 2003 : GROUP ART UNIT: 1636

FOR: CELL DIVISION-VISUALIZED: CELL AND METHOD OF PRODUCTION OF THE SAME, METHOD OF DETECTION OF FLUORESCENCE, METHOD OF EVALUATION OF INFLUENCE UPON CELL DIVISION, AND METHOD OF SCREENING

DECLARATION UNDER 37 C.F.R. 1.132

COMMISSIONER FOR PATENTS
ALEXANDRIA, VIRGINIA 22313

SIR:

Now comes Kenji SUGIMOTO who deposes and states:

1. That I am a joint inventor of the present invention.

2. That Kenji SUGIMOTO, Takeshi URANO and Makoto TACHIBANA are the joint inventors of the present invention.

3. That SUGIMOTO et al., Cell Structure and Function, vol. 27, pages 457-467 (December, 2002) describes the work of the present joint inventors who are Kenji SUGIMOTO, Takeshi URANO and Makoto TACHIBANA.

4. That Hitomi ZUSHI, Kimiko INOUE, Hiroaki TASAKA and Masaya DOTSU, who are listed as coauthors on Cell Structure and Function, vol. 27, pages 457-467 (December, 2002)

did not contribute to the present invention and/or were working under the direction of the present inventors.

5. That SUGIMOTO et al., Molecular Biology of the Cell, vol. 13, pages 50a-51a, Abstract 282 (2002) also describes the work of the present joint inventors who are Kenji SUGIMOTO, Takeshi URANO and Makoto TACHIBANA.

6. That M. DOTSU who is listed as an author on SUGIMOTO et al., Molecular Biology of the Cell, vol. 13, pages 50a-51a, did not contribute to the present invention and/or was working under the direction of the present inventors.

7. The undersigned petitioner declares further that all statements made herein of his own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of this application or any patent issuing thereon.

8. Further deponent saith not.

Signature Kenji Sugimoto

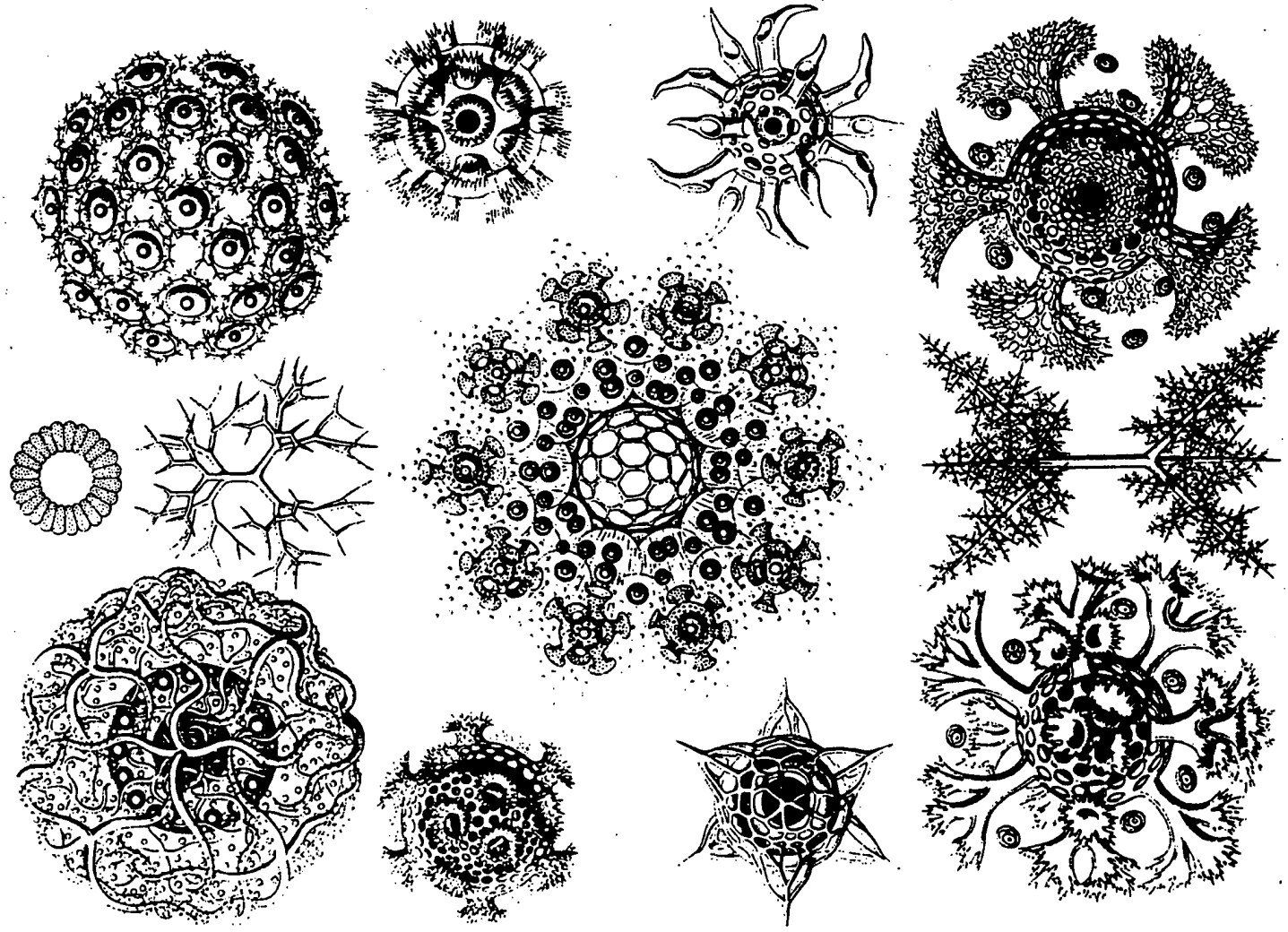
Date July 27, 2005

Molecular Biology of the Cell

Supplement to Molecular Biology of the Cell Volume 13 Nov 2002

Abstracts

42nd American Society for Cell Biology Annual Meeting
December 14–18, 2002, San Francisco, CA



different interactions of centrioles with the mitotic spindle, and by observing cell division defects as a function of the initial centriole number, we have obtained evidence that most defects caused by abnormal centriole numbers depend on effects on the mitotic spindle.

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Controlling Centrosome Number: Evidence for a Block to Centrosome Over-duplication

C. Wong, T. Stearns; Department of Biological Sciences, Stanford University, Stanford, CA

The centrosome is the microtubule organizing center of animal cells. Cells typically have a single centrosome in G1, which duplicates during S phase. The duplicated centrosomes organize the bipolar mitotic spindle in M phase, and defects in centrosome duplication can lead to the formation of monopolar or multipolar spindles and missegregation of chromosomes. Such defects are commonly observed in cancer cells, suggesting that maintenance of centrosome number is essential to genome stability. It is unclear whether centrosome number is controlled simply by periodic activation of initiation of duplication, or by an inhibitory mechanism to prevent over-duplication. Here we address this question using a cell fusion approach in which unduplicated G1 centrosomes and duplicated G2 centrosomes are combined in the same cytoplasm. We find that only one of the three centrosomes in such fused cells duplicates. Thus the common cytoplasm has the factors to carry out centrosome duplication, yet only one of the centrosomes responds, revealing the presence of a mechanism to prevent centrosome over-duplication.

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Role of p53 in Regulation of Centrosome Duplication

P. Tarapore, K. Fukasawa; Cell Biology, University of Cincinnati, Cincinnati, OH

Centrosome amplification has been found to be associated with chromosomal instability during tumor development. The presence of excess copies of centrosomes increase the frequency of mitotic defects, leading to unbalanced chromosome transmission to daughter cells. Loss or mutational inactivation of p53 tumor suppressor protein leads to abnormal amplification of centrosomes. Centrosome duplication is controlled through two major regulatory mechanisms. The first involves the correct timing for initiation of centrosome duplication, which has to occur in coordination with DNA replication, and the second is suppression of re-duplication of centrosomes. We have found that p53 is involved in both regulations. In p53+/+ MEFs, initiation of centrosome duplication is tightly coupled with initiation of DNA replication. In contrast, in p53-/- MEFs, centrosomes initiate duplication in early G_1 much before S-phase entry. When cells are arrested in G_1/S phase, which is permissive for centrosome duplication, centrosomes continue to duplicate in p53-/- MEFs, resulting in generation of multiple copies of centrosomes. In contrast, no centrosome duplication is observed in p53+/+ MEFs. However, both controls are restored in p53-/- MEFs expressing exogenous wild-type p53, indicating that p53 is directly and actively involved in the regulation of centrosome duplication. The majority of p53-mediated functions are attributed to its transactivation activity. Similarly, we have found that p53 controls centrosome duplication via transactivation-dependent mechanisms. In its transactivation-dependent control, $p21^{Waf1/Cip1}$ acts as a major effector, likely guarding against untimely activation of CDK2/cyclin E kinase, hence ensuring the coordinated initiation of centrosome and DNA duplication. In addition to its transactivation-dependent control, p53 also exerts a transactivation-independent mechanism to regulate centrosome duplication. We have found that p53 exerts its transactivation-independent control through direct physical binding to unduplicated centrosomes. Moreover, the ability to bind to unduplicated centrosomes is controlled by phosphorylation on p53-Ser 315.

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Differential Effects of Specific p53 Mutations on Centrosome Structure and Function

W. L. Lingle, V. C. Negron, S. L. Barrett, C. E. Gibson; Experimental Pathology, Mayo Clinic, Rochester, MN

Our recent studies of p53 mutations in human breast cancer demonstrated that tumors with mutant p53 tend to have a significantly greater capacity to nucleate microtubules than do tumors with wild type p53. Microtubule nucleation is a major function of the centrosome during both interphase and mitosis. To further our understanding of the relationship between p53 and centrosome function, we have created two adenoviral vectors, M114 and M72, containing two different p53 mutations. We chose a specific p53 mutation present in a tumor with a microtubule nucleating capacity 10 fold higher than that of normal breast epithelial cells (M114) and another mutation from a tumor with a 2 fold higher capacity (M72). Both tumors had high levels of chromosomal instability as determined by FISH using centromeric probes to chromosomes 3, 7, and 17. We used the adenoviral vectors to deliver the mutant p53 to cultured human mammary epithelial cells (BioWhitaker, Inc.). These cells, which were derived from normal breast epithelium, senesce after 15 to 18 passages. Infection rates of greater than 90% were achieved with the adenoviral vectors. Cells infected with both M114 and M72 had higher frequencies of multinucleate cells and cells with amplified centrosomes than did cells infected with a GFP-expressing control virus. However, the cells infected with M114 had a much higher frequency of multipolar mitoses than did the cells infected with M72 or the control virus. M114 infected cells also had a much faster in vivo microtubule regrowth rate than cells infected with M72 or the control virus. These results indicate that specific p53 mutations have different effects on centrosome structure and function and that mutant p53 can affect the microtubule cytoskeleton.

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Centrosome abnormalities and chromosome instability occur together in precancerous lesions

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Centrosomes play critical roles in processes that ensure proper segregation of chromosomes and maintain the genetic stability of human cells. They contribute to mitotic spindle organization and regulate aspects of cytokinesis and cell cycle progression. We and others have shown that centrosomes are abnormal in most aggressive carcinomas. Moreover, centrosome defects have been implicated in chromosome instability and loss of cell cycle control in invasive carcinoma. Others have suggested that centrosome defects only occur late in tumorigenesis and may not contribute to early steps of tumor development. To address this issue, we examined early human carcinoma in-situ lesions for centrosome defects and chromosome instability. We found that a significant fraction of precursor lesions to some of the most common human cancers had centrosome defects including in situ carcinomas of the uterine cervix, prostate and female breast. Moreover, centrosome defects occurred together with mitotic spindle defects, chromosome instability and high cytologic grade among these lesions. Finally, a cell line established from an in situ carcinoma of the prostate showed a higher rate of aberrant centrosomes, multipolar spindles and chromosome instability than an isogenic line derived from the same organ. Our findings demonstrate that centrosome defects occur concurrently with chromosome instability and cytologic changes in the earliest identifiable step in human cancer. Our results suggest that centrosome defects may contribute to the earliest stages of cancer development through the generation of chromosome instability. This, together with ongoing structural changes in chromosomes, could accelerate accumulation of alleles carrying pro-oncogenic mutations and loss of alleles containing wild-type tumor suppressor genes and thus accelerate the genomic changes characteristic of carcinoma, the most prevalent human cancer.

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Knockdown of Centrin-2 Arrests Centriole Duplication and Activates a Centrosome-based Cell Cycle Checkpoint

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Centrosomes contain a pair of centrioles that normally duplicate once during the cell cycle to give rise to two mitotic spindle poles, each containing one old and one new centriole. The centrins are small calcium-binding proteins that are ubiquitous centrosome components. Humans have three centrin genes: Cetn-1, Cetn-2, and Cetn-3. Centrin-1 is expressed exclusively in male germ cells. Centrins-2 and -3 are expressed in somatic cells. Centrin-2 is a centriole protein and centrin-3 localizes to the pericentriolar material that surrounds the centrioles. Several emerging lines of evidence suggest that cell cycle pathways converge on the centrosome and implicate this organelle in the control of cell cycle progression in addition to its function as a microtubule-organizing center. Here, we establish an essential function for the centrosome protein – centrin-2 - in this process through its role in centriole duplication. Using RNA interference, we demonstrate that 'knockdown' of centrin-2, a protein of centrioles, results in failure of centrioles to duplicate during the cell cycle in HeLa cells. Following inhibition of centrin synthesis, the pre-existing pair of centrioles separate and functional bipolar spindles form with only one centriole at each spindle pole. Centriole dilution results from the ensuing cell division and daughter cells are 'born' with only a single centriole. Remarkably, these uni-centriolar daughter cells may go on to complete a second mitosis in which spindle microtubules converge onto unusually broad spindle poles and where cell division results in daughter cells containing either one or no centrioles at all. Cells thus denuded of the mature or both centrioles rapidly succumb to cell death, presumably through the activation of the apoptotic pathway. These observations suggest the existence of a centrosome-based cell cycle checkpoint that functions to monitor centriole number.

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Molecular dynamics in living mitotic cells of histone H3, centrosomal kinase Aurora-A and nuclear membrane protein importin alpha differentially expressed as fusions to cyan, green and red fluorescent proteins

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We constructed a human stable cell-line in which histone H3, Aurora-A kinase, and importinα were differentially expressed as fusions to cyan, green and red fluorescent proteins (CFP, GFP and DsRed). Their molecular behavior in living mitotic cells was extensively characterized by an advanced timelapse image analyzing system with a highly sensitive CCD camera. To follow the movement

throughout the mitosis, X-Y images were collected with optical sections in 2μm depth and with 2min intervals. In G2 phase, duplicated centrosomal dots of GFP-Aurora-A separate and move to the opposite poles. In prophase, the centrosomal dots approach closer and the nuclear membrane beneath them, identified by DsRed-importinα, becomes thick and invaginated, resulting in a "dumb-bell" shape with condensed chromatin on the inside wall of nuclear membrane. As the importinα membrane further shrinks and disappears, mitotic chromosomes are excluded from the nucleus and the centrosomal dots grow rapidly and transform into a spindle-like structure. Prometaphase continues until the chromosomes are appropriately arranged at a spindle equator. Anaphase onset is easily identified when the sister chromatids start segregation. It only takes 4-6 min for them to reach the poles. The membrane structure of importinα reappears on the surface of condensed chromatin within 10 min after anaphase onset. Interestingly, part of Aurora-A accumulate in spindle midzone and then localize to midbody in the completion of cytokinesis, while the maturated centrosomal dots gradually decrease in size and return to the surface of the newly formed small sister nuclei, which gradually enlarge in size in G1. These results clearly showed that CFP-histone H3, GFP-Aurora-A and Ds-Red-importinα are excellent markers for the live image analysis of mammalian cell divisions.

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***Drosophila* Checkpoint Kinase 2 Regulates Centrosome Function and Spindle Assembly in Response to Genotoxic Stress**

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In syncytial *Drosophila* embryos, DNA damage or incomplete DNA replication at the onset of mitosis triggers loss of microtubule nucleation by the centrosomes, anastral spindle assembly, and chromosome segregation failures (Sibon *et al.* 2000, Nature Cell Biology, 2:90-95). We show that centrosome inactivation is induced by a wide range of DNA damaging agents and replication inhibitors, and by direct injection of double-strand breaks or single-stranded DNA. By contrast, injection of supercoiled DNA does not efficiently induce centrosome defects. Centrosome inactivation and chromosome segregation failures thus appear to be triggered by a broad spectrum of mutagenic DNA lesions. Previous studies indicate that centrosome inactivation is accompanied by loss of γTuRC components from a core centrosome structure. Here we show that DNA damage and replication defects also disrupt localization of Polo kinase, Asp, and *Drosophila* transforming acidic coiled-coil protein (DTACC) to the centrosome, suggesting that this response targets multiple centrosomal proteins, and not just microtubule nucleation factors. Checkpoint kinase 2 (Chk2) is a conserved component of the DNA damage cell cycle checkpoint pathway. We find that a null mutation in the *Drosophila* Checkpoint kinase 2 gene blocks DNA damage/incomplete replication-induced centrosome inactivation. Immunolocalization studies demonstrate that DmChk2 is not localized during mitosis in control embryos. However, DmChk2 accumulates at centrosomes and along spindle microtubules following DNA damage, suggesting that it directly modifies spindle components. In other systems, Chk2 is downstream of the ATM (Ataxia Telangiectasia-Mutated) tumor suppressor in the DNA damage checkpoint pathway. Our preliminary results suggest that the *Drosophila* ATM homologue is required for DNA damage induced centrosome inactivation. We propose that the ATM/Chk2 pathway disrupts centrosome function and spindle assembly when DNA lesions persist into mitosis, thus limiting propagation of mutations caused by genotoxic stress.

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Myosin and actin are required for centrosome separation during prophase

I. Rosenblatt,[1] B. Baum,[2] J. Blyth,[1] L. P. Cramer[1] ; [1] Laboratory for Molecular Cell Biology, MRC, London, United Kingdom, [2] University College London, Ludwig Institute, London, United Kingdom

Microtubules and their motor proteins are essential components of mitotic spindles, but the role of actin and myosin in mitosis has historically been restricted largely to cytokinesis. Surprisingly, we find that myosin and actin are required for centrosome separation in 4/4 cell lines (mouse hybridoma cells, Swiss 3T3 fibroblast, and PtK2 and MDCK epithelial cells). We disrupted either actin polymerization with Latrunculin A or Cytochalasin D or myosin contractility with the Rho kinase inhibitor Y-27632 or the myosin light chain kinase inhibitor ML-9 and analyzed spindle assembly in either live or fixed cells. Timelapse movies of PtK2 cells expressing GFP-tubulin showed that disruption of either actin or myosin halts the separation of duplicated centrosomes around the nucleus and prevents microtubule asters from migrating to opposite sides of chromatin. Instead, the centrosomes remain on one side of the chromatin or extra asters spontaneously nucleate around the chromatin. The result in both cases is the formation of abnormal spindle structures. In over 1000 spindles analyzed in PtK2 cells treated with either Latrunculin A or Y-27632, over 50% of cells formed abnormal spindles, compared to 6% in DMSO-treated control cells. Strikingly, similar results were obtained when RNAi was used to disrupt actin and myosin in *Drosophila* S2R+ cells. Finally, depolymerization of actin filaments with Latrunculin A prevented the interactions of astral microtubule with the cortex, whereas, inhibition of myosin activity had no effect. Our results suggests that myosin contractility within the actin cortex helps segregate centrosomes via astral microtubule connections to the cortex.

Cytoskeletal Organization I (285-299)

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An inhibitor of PLC phosphorylation in PKA-mediated parietal cell secretion

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Within the gastric parietal cell, activation of the PKA cascade triggers the translocation and fusion of H/K-ATPase (HK) containing vesicles to the apical membrane, resulting in HCl secretion. The involvement of actin and the cytoskeletal-linker protein ezrin in this process is suggested by the fact that drugs that inhibit acid secretion also disrupt the membrane localization of either actin or ezrin. In the rabbit parietal cell, stimulation of either the PLC pathway (carbachol) or the PKA pathway (histamine) leads to secretion, suggesting there may be some convergence of the two pathways. We have been studying the action of the kinase inhibitor ME3407, which reduces secretion and effectively releases ezrin from its normal membrane-bound location [AJP 35: G1122]. Gastric glands were treated with cimetidine (resting), histamine (stimulated), or histamine plus ME3407. Proteins were then run on 2D gels, Western transferred, and probed with various antibodies. At present, we do not see a consistent change in the phosphorylation state of ezrin with the addition of ME3407. Using an antibody specific for PKC-phosphorylation sites, results show that histamine treatment increases phosphorylation at PKC sites on certain proteins, even though histamine signals through a PKA-mediated pathway. Furthermore, the same antibody shows that ME3407 dramatically reduced the phosphorylation of a ~70 kDa protein. We have also used a cultured parietal cell system to monitor the timecourse of HK translocation by immunofluorescence. ME3407 prevents HK translocation when added prior to stimulants, in agreement with prior work in gastric glands. Addition of ME3407 to partially stimulated cells prevents further translocation of HK, indicating that the drug inhibits a process associated with vesicle fusion. These data lend further evidence to the theory that histamine-stimulated acid secretion also involves the PKC pathway.

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Identification of a Novel Signaling Pathway Required for Uptake of Bacterial Pathogens

E. A. Burton, A. Pendergast; Pharmacology & Cancer Biology, Duke University, Durham, NC

The ability of bacterial pathogens to enter non-phagocytic cells is an essential step for virulence. The process of bacterial entry is highly regulated, requiring both pathogen and host cell factors. Many pathogens utilize the cellular signaling pathways that regulate actin cytoskeletal dynamics, such as the Rho family GTPases. The activation of the Rho family GTPases has been demonstrated to be required for the uptake of many pathogens, including *Salmonella typhimurium* and *Shigella flexneri*. This manipulation of cellular signaling pathways by pathogens provides a unique model system to study the pathways regulating reorganization of the actin cytoskeleton. Using null cells and pharmacological inhibitors, we have identified a novel signaling pathway required for the uptake of bacterial pathogens. Experiments to determine the role of this pathway in the activation of Rho GTPases and in cytoskeletal reorganization are currently in progress. These data support a role for this signaling pathway in both cytoskeletal dynamics and in bacterial pathogenesis. (E.A.B. is a Fellow of the Leukemia & Lymphoma Society #5912-01).

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***Drosophila* ERM Protein Moesin Functions Antagonistically to the Small GTPase Rho in Epithelial Cells**

O. Nikiforova; Biology/DCMB, Duke University, Durham, NC

Because of their ability to associate with transmembrane proteins and actin filaments, the ERM (Ezrin, Radixin, Moesin) proteins have been thought to act as cross-linkers between the cortical cytoskeleton and the plasma membrane. *In vivo* analyses of ERM function that could test this view have been impeded by the redundancies between these three proteins in mammals . In contrast, *Drosophila* has only one ERM gene, *Moesin*, enabling us to study ERM function *in vivo*. Phenotypic analysis of a *Moesin* loss-of-function allele shows that Moesin promotes cortical actin assembly and apical-basal polarity in epithelial cells. *Moesin*$^{-}$ cells undergo an abnormal epithelial-to-mesenchymal transition, accompanied by loss of junctional markers and apico-basal polarity, and adopt invasive migratory behavior. At the same time, filamentous actin undergoes dramatic redistribution and becomes depleted from the apical region. Strikingly similar phenotypes are observed when the function of the small GTPase Rho is upregulated in epithelial cells. Genetic interactions with *Rho* and Rho pathway components show that Moesin facilitates epithelial morphology not by cross-linking the plasma membrane to the cytoskeleton, but rather by antagonizing Rho pathway activity. Furthermore, because ERM proteins are known downstream effectors of Rho signaling, our results suggest the existence of a negative feedback loop between ERM proteins and the Rho pathway. This negative feedback mechanism may be important in inhibiting migratory and invasive cellular behaviors that characterize metastatic cells. We are in the process of

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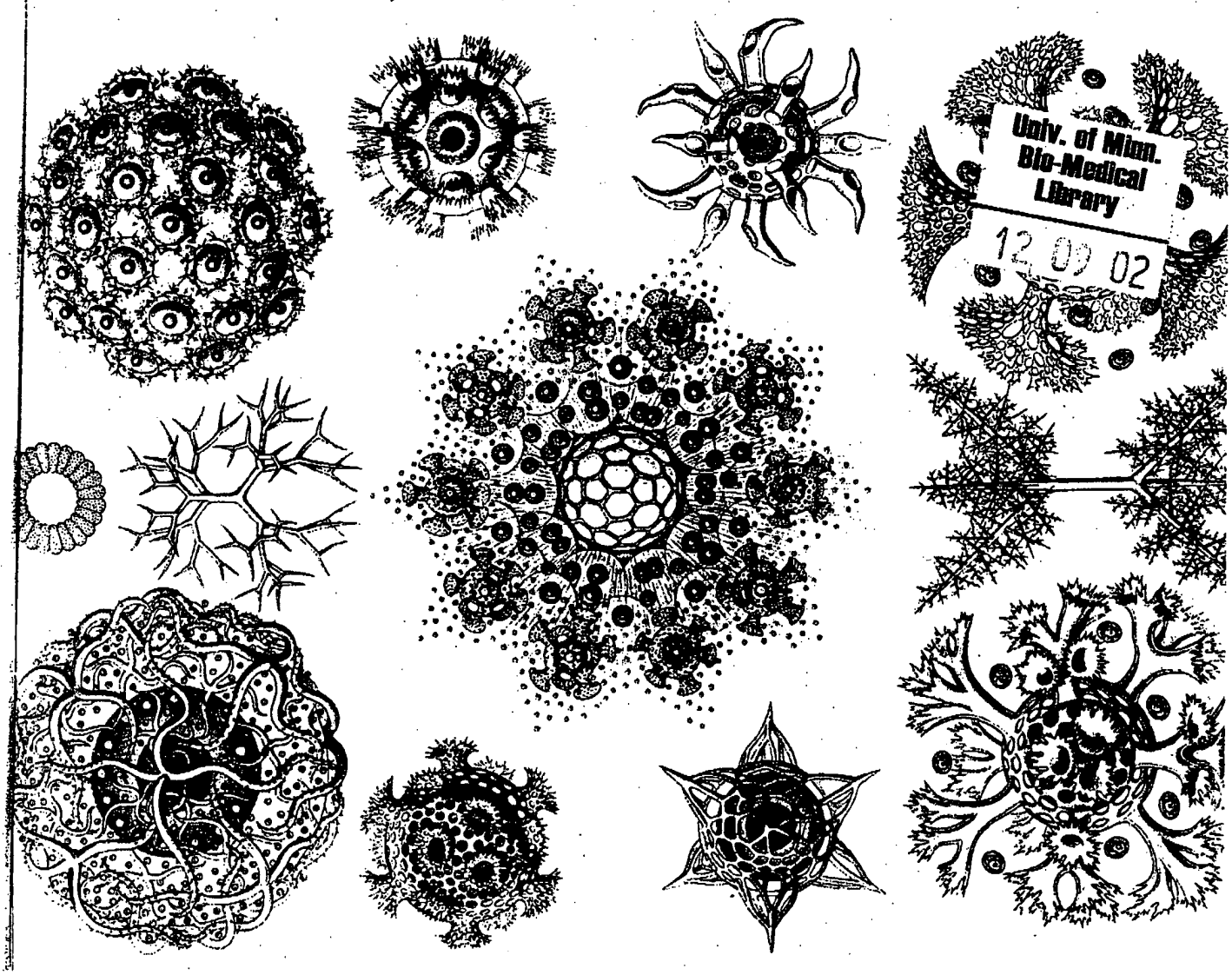

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C. Wong, T. Stearns; Department of Biological Sciences, Stanford University, Stanford, CA

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G. Pihan,[1] S. J. Doxsey[2]; [1] Pathology, University of Massachusetts, Worcester, MA, [2] Molecular Medicine, University of Massachusetts, Worcester, MA

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We constructed a human stable cell-line in which histone H3, Aurora-A kinase, and importin were differentially expressed as fusions to cyan, green and red fluorescent proteins (CFP, GFP and DsRed). Their molecular behavior in living mitotic cells was extensively characterized by an advanced timelapse image analyzing system with a highly sensitive CCD camera. To follow the movement

throughout the mitosis, X-Y images were collected with optical sections in 2μm depth and with 2min intervals. In G2 phase, duplicated centrosomal dots of GFP-Aurora-A separate and move to the opposite poles. In prophase, the centrosomal dots approach closer and the nuclear membrane beneath them, identified by DsRed-importinα, becomes thick and invaginated, resulting in a "dumb-bell" shape with condensed chromatin on the inside wall of nuclear membrane. As the importinα membrane further shrinks and disappears, mitotic chromosomes are excluded from the nucleus and the centrosomal dots grow rapidly and transform into a spindle-like structure. Prometaphase continues until the chromosomes are appropriately arranged at a spindle equator. Anaphase onset is easily identified when the sister chromatids start segregation. It only takes 4-6 min for them to reach the poles. The membrane structure of importinα reappears on the surface of condensed chromatin within 10 min after anaphase onset. Interestingly, part of Aurora-A accumulate in spindle midzone and then localize to midbody in the completion of cytokinesis, while the maturated centrosomal dots gradually decrease in size and return to the surface of the newly formed small sister nuclei, which gradually enlarge in size in G1. These results clearly showed that CFP-histone H3, GFP-Aurora-A and Ds-Red-importinα are excellent markers for the live image analysis of mammalian cell divisions.

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***Drosophila* Checkpoint Kinase 2 Regulates Centrosome Function and Spindle Assembly in Response to Genotoxic Stress**

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In syncytial *Drosophila* embryos, DNA damage or incomplete DNA replication at the onset of mitosis triggers loss of microtubule nucleation by the centrosomes, anastral spindle assembly, and chromosome segregation failures (Sibon *et al.* 2000, Nature Cell Biology, 2:90-95). We show that centrosome inactivation is induced by a wide range of DNA damaging agents and replication inhibitors, and by direct injection of double-strand breaks or single-stranded DNA. By contrast, injection of supercoiled DNA does not efficiently induce centrosome defects. Centrosome inactivation and chromosome segregation failures thus appear to be triggered by a broad spectrum of mutagenic DNA lesions. Previous studies indicate that centrosome inactivation is accompanied by loss of γTuRC components from a core centrosome structure. Here we show that DNA damage and replication defects also disrupt localization of Polo kinase, Asp, and *Drosophila* transforming acidic coiled-coil protein (DTACC) to the centrosome, suggesting that this response targets multiple centrosomal proteins, and not just microtubule nucleation factors. Checkpoint kinase 2 (Chk2) is a conserved component of the DNA damage cell cycle checkpoint pathway. We find that a null mutation in the *Drosophila* Checkpoint kinase 2 gene blocks DNA damage/incomplete replication-induced centrosome inactivation. Immunolocalization studies demonstrate that DmChk2 is not localized during mitosis in control embryos. However, DmChk2 accumulates at centrosomes and along spindle microtubules following DNA damage, suggesting that it directly modifies spindle components. In other systems, Chk2 is downstream of the ATM (Ataxia Telangiectasia-Mutated) tumor suppressor in the DNA damage checkpoint pathway. Our preliminary results suggest that the *Drosophila* ATM homologue is required for DNA damage induced centrosome inactivation. We propose that the ATM/Chk2 pathway disrupts centrosome function and spindle assembly when DNA lesions persist into mitosis, thus limiting propagation of mutations caused by genotoxic stress.

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Myosin and actin are required for centrosome separation during prophase

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Microtubules and their motor proteins are essential components of mitotic spindles, but the role of actin and myosin in mitosis has historically been restricted largely to cytokinesis. Surprisingly, we find that myosin and actin are required for centrosome separation in 4/4 cell lines (mouse hybridoma cells, Swiss 3T3 fibroblast, and PtK2 and MDCK epithelial cells). We disrupted either actin polymerization with Latrunculin A or Cytochalasin D or myosin contractility with the Rho kinase inhibitor Y-27632 or the myosin light chain kinase inhibitor ML-9 and analyzed spindle assembly in either live or fixed cells. Timelapse movies of PtK2 cells expressing GFP-tubulin showed that disruption of either actin or myosin halts the separation of duplicated centrosomes around the nucleus and prevents microtubule asters from migrating to opposite sides of chromatin. Instead, the centrosomes remain on one side of the chromatin or extra asters spontaneously nucleate around the chromatin. The result in both cases is the formation of abnormal spindle structures. In over 1000 spindles analyzed in PtK2 cells treated with either Latrunculin A or Y-27632, over 50% of cells formed abnormal spindles, compared to 6% in DMSO-treated control cells. Strikingly, similar results were obtained when RNAi was used to disrupt actin and myosin in *Drosophila* S2R+ cells. Finally, depolymerization of actin filaments with Latrunculin A prevented the interactions of astral microtubule with the cortex, whereas, inhibition of myosin activity had no effect. Our results suggests that myosin contractility within the actin cortex helps segregate centrosomes via astral microtubule connections to the cortex.

Cytoskeletal Organization I (285-299)

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An inhibitor of PLC phosphorylation in PKA-mediated parietal cell secretion

D. A. Ammar, K. Kao, A. Cheng, J. G. Forte; Molecular & Cell Biology, *University of California, Berkeley, CA*

Within the gastric parietal cell, activation of the PKA cascade triggers the translocation and fusion of H/K-ATPase (HK) containing vesicles to the apical membrane, resulting in HCl secretion. The involvement of actin and the cytoskeletal-linker protein ezrin in this process is suggested by the fact that drugs that inhibit acid secretion also disrupt the membrane localization of either actin or ezrin. In the rabbit parietal cell, stimulation of either the PLC pathway (carbachol) or the PKA pathway (histamine) leads to secretion, suggesting there may be some convergence of the two pathways. We have been studying the action of the kinase inhibitor ME3407, which reduces secretion and effectively releases ezrin from its normal membrane-bound location [AJP 35: G1122]. Gastric glands were treated with cimetidine (resting), histamine (stimulated), or histamine plus ME3407. Proteins were then run on 2D gels, Western transferred, and probed with various antibodies. At present, we do not see a consistent change in the phosphorylation state of ezrin with the addition of ME3407. Using an antibody specific for PKC-phosphorylation sites, results show that histamine treatment increases phosphorylation at PKC sites on certain proteins, even though histamine signals through a PKA-mediated pathway. Furthermore, the same antibody shows that ME3407 dramatically reduced the phosphorylation of a ~70 kDa protein. We have also used a cultured parietal cell system to monitor the timecourse of HK translocation by immunofluorescence. ME3407 prevents HK translocation when added prior to stimulants, in agreement with prior work in gastric glands. Addition of ME3407 to partially stimulated cells prevents further translocation of HK, indicating that the drug inhibits a process associated with vesicle fusion. These data lend further evidence to the theory that histamine-stimulated acid secretion also involves the PKC pathway.

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Identification of a Novel Signaling Pathway Required for Uptake of Bacterial Pathogens

E. A. Burton, A. Pendergast; Pharmacology & Cancer Biology, Duke University, Durham, NC

The ability of bacterial pathogens to enter non-phagocytic cells is an essential step for virulence. The process of bacterial entry is highly regulated, requiring both pathogen and host cell factors. Many pathogens utilize the cellular signaling pathways that regulate actin cytoskeletal dynamics, such as the Rho family GTPases. The activation of the Rho family GTPases has been demonstrated to be required for the uptake of many pathogens, including *Salmonella typhimurium* and *Shigella flexneri*. This manipulation of cellular signaling pathways by pathogens provides a unique model system to study the pathways regulating reorganization of the actin cytoskeleton. Using null cells and pharmacological inhibitors, we have identified a novel signaling pathway required for the uptake of bacterial pathogens. Experiments to determine the role of this pathway in the activation of Rho GTPases and in cytoskeletal reorganization are currently in progress. These data support a role for this signaling pathway in both cytoskeletal dynamics and in bacterial pathogenesis. (E.A.B. is a Fellow of the Leukemia & Lymphoma Society #5912-01).

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***Drosophila* ERM Protein Moesin Functions Antagonistically to the Small GTPase Rho in Epithelial Cells**

O. Nikiforova; Biology/DCMB, Duke University, Durham, NC

Because of their ability to associate with transmembrane proteins and actin filaments, the ERM (Ezrin, Radixin, Moesin) proteins have been thought to act as cross-linkers between the cortical cytoskeleton and the plasma membrane. *In vivo* analyses of ERM function that could test this view have been impeded by the redundancies between these three proteins in mammals . In contrast, *Drosophila* has only one ERM gene, *Moesin*, enabling us to study ERM function *in vivo*. Phenotypic analysis of a *Moesin* loss-of-function allele shows that Moesin promotes cortical actin assembly and apical-basal polarity in epithelial cells. *Moesin*$^{-}$ cells undergo an abnormal epithelial-to-mesenchymal transition, accompanied by loss of junctional markers and apico-basal polarity, and adopt invasive migratory behavior. At the same time, filamentous actin undergoes dramatic redistribution and becomes depleted from the apical region. Strikingly similar phenotypes are observed when the function of the small GTPase Rho is upregulated in epithelial cells. Genetic interactions with *Rho* and Rho pathway components show that Moesin facilitates epithelial morphology not by cross-linking the plasma membrane to the cytoskeleton, but rather by antagonizing Rho pathway activity. Furthermore, because ERM proteins are known downstream effectors of Rho signaling, our results suggest the existence of a negative feedback loop between ERM proteins and the Rho pathway. This negative feedback mechanism may be important in inhibiting migratory and invasive cellular behaviors that characterize metastatic cells. We are in the process of

・HP address of American society for cell biology : https://www.ascb.org/
・Mail address for inquiry : ascbinfo@ascb.org

We show you the actual mail contents as follows:

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5)
—Original Message—
From: Rachel Altemus [mailto:RALTEMUS@ascb.org]
Sent: Tuesday, May 31, 2005 11:32 PM
To: ok.?¶ @-Ì"Í
Subject: RE: The date of issue for 42nd ASCB Annual Meeting Abstracts

For the exact mailing date you may proceed with November 27, 2002.

Rachel Altemus

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4)
>>> <shigenori.oka@nagase.co.jp> 5/26/2005 9:23:30 PM >>>

Dear Rachel Altemus:

I thank you for your reply.
May I ask you a question ?

I got two replys from you and Mr Raymond L. Evemgam, Jr.
His reply is " The issue date was: November 27, 2002".
What is the differences between the two (Nov. 1 and Nov. 27) ?

Your attention will be appreciated.

Sincerely yours,

Shigenori Oka

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3)
—Original Message—
From: Rachel Altemus [mailto:RALTEMUS@ascb.org]
Sent: Friday, May 27, 2005 1:11 AM
To: ok.****
Subject: Re: The date of issue for 42nd ASCB Annual Meeting Abstracts

The official publication for Vol. 13 Supplement is November 1, 2002.

Rachel Altemus

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2)
----Original Message----
From: Ray Evernqam [mailto:revernqam@ascb.org]
Sent: Thursday, May 26, 2005 10:30 PM
To: ok.****
Subject: Re: The date of issue for 42nd ASCB Annual Meeting Abstracts

The issue date was: November 27, 2002.

Raymond L. Evernqam, Jr.
Director of Publications
American Society for Cell Biology
8120 Woodmont Avenue, Suite 750
Bethesda, MD 20814-2762

Phone: 301-347-9300
Fax: 301-347-9310

****

1)
>>> <shigenori.oka@nagase.co.jp> 5/26/2005 6:32:12 AM >>>
Dear Sirs,

The information about the date of issue for 42nd ASCB Annual Meeting Abstracts vol.13 (Dec. 14-18, 2002, San Francisco) you may furnish us will be greatly appreciated.

(I would like to get the information about publication date of 42nd ASCB Annual Meeting Abstracts. Could you give me the information.)

Yours faithfully,

****

Please acknowledge the safe receipt of this letter by return facsimile.
With best regards,

Yours, very sincerely

KOJIMA INTERNATIONAL PATENT OFFICE
Yoshinori Hagino
Yoshinori Hagino